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(54) Title: METHOD FOR RETROVIRAL VECTOR INSERTION IN FISH

(57) Abstract

A method for stably introducing exogenous DNA into the germ line of a fish is described. A fish embryo is provided. A retroviral vector is introduced into a cell of the fish embryo, and the fish embryo is allowed to develop into a fish. Stable chromosomal insertions of the retroviral vector are screened for. Transgenic fish, cells and cell lines are also provided.

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METHOD FOR RETROVIRAL VECTOR INSERTION IN FISH

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Field of the Invention

This invention relates to a method for making stable retroviral vector insertions into the germ line of a fish, and to transgenic fish.

Background of the Invention

In recent years the zebrafish, Danio rerio, has become a popular model system for vertebrate developmental studies because it offers the opportunity to combine classical genetic analysis, including large scale mutagenesis, with an easily accessible and manipulable embryo. Genetic studies of the zebrafish benefit from the 2-3 month generation time, the ability of females to routinely lay hundreds of eggs, and the small size of the adults. Embryological studies benefit from the large, transparent embryos, detailed fate maps, and the fact that single identified cells can be studied in living embryos.

At present, genetic screens are typically done by mutagenizing fish genomes with chemical mutagens, e.g., N-ethyl-N-nitrourea, or gamma rays. The mutated genes resulting from such procedures are very difficult to clone because of the current absence of high-resolution genetic and physical maps of the zebrafish genome, and the derth of available probes.

Retroviruses have been used to infect mammals, e.g., mice, and have been shown to integrate into the genomes of such infected cells. They have been used in studies of mouse development, gene therapy, cell lineage and insertional mutagenesis. The application of retroviral vector technology to the zebrafish system has not been feasible due in part to the limited host range of the standard vectors. It has recently been reported that a pseudotyped retroviral vector can infect cultured fish cells. Burns et al., Proc. Nat'l Acad. Sci. USA, 90: 8033-8037 (1993).

Summary of the Invention

It is an object of the invention to make stable retroviral vector insertions in the germ line of a fish.

It is another object of the invention to obtain germ line transmission of an integrated provirus.

It is yet another object of the invention to make transgenic fish using retroviruses.

It is yet another object of the invention to make stable insertional mutations in fish.

It is yet another object of the invention to stably introduce a desired characteristic into a fish.

Still another object of the invention is to be able to readily clone mutated genes by using inserted retroviral vector sequences as a probe.

According to the invention, a method for stably introducing exogenous DNA into the germ line of a fish, e.g., zebrafish, is provided. A fish embryo having cells is provided. A retroviral vector is introduced into a cell of the fish embryo. The fish embryo is permitted to develop into a fish. Stable chromosomal insertion of the retroviral vector is screened for, e.g., in the fish embryo, in the fish, or in the F1 progeny of the fish.

The retroviral vector can be, e.g., a pseudotyped virus having a host range so as to be able to enter the cell of the fish embryo and integrate into the genome of a cell. In certain embodiments, the retroviral vector is packaged in particles having an envelope from a first virus, e.g., a rhabdovirus, e.g, vesicular stomatitis virus. Preferably, the retroviral vector has at least one viral gene derived from a second virus, e.g., a retrovirus, e.g., Moloney murine leukemia virus. In other embodiments, the retroviral vector also has a reporter gene, e.g., the gene encoding lacZ, tyrosinase or green fluorescent protein. In yet other embodiments, the retroviral vector carries an exogenous DNA sequence to impart a desired characteristic.

Variations of this method of this invention include introducing the retroviral vector, e.g., by injection, into a fish embryo, e.g., the blastoderm, into a blastula stage embryo, or into an embryo having about 1 to about 1000 cells, about 1000 to about 2000 cells, about 2000 to about 4000 cells, about 4000 to about 8000 cells, or greater than about 8000 cells. In certain embodiments the retroviral vector is introduced into the cell in the presence of polybrene, preferably at about 2 ?m?g/ml to about 20 ?m?g/ml, and most preferably at about 8 ?m?g/ml.

In certain embodiments, introduction of the retroviral vector creates a mutation in the fish, and in other embodiments, insertion of the retroviral vector provides the fish with a desired characteristic.

Another aspect of the invention is a method for stably introducing exogenous DNA into the germ line of a fish by providing a fish having cells, introducing a retroviral vector into a cell of the fish, and screening for stable chromosomal insertion of the retroviral vector.

Another aspect of the invention is a method for making a stable insertional mutation in a fish in which a retroviral vector is introduced into a cell in a fish embryo, the fish embryo is permitted to devel p into a fish, and stable chromosomal insertion of the retroviral vector and the presence of a

mutation are screened for.

Another aspect of the invention is a method for treating a fish cell in an embryo so as to cause an insertion of a retroviral vector in a chromosome of the fish cell. A retroviral vector is introduced into a fish cell in an embryo, and the fish cell, or a descendent of the fish cell, is screened for insertion of the retroviral vector into a chromosome of the fish cell.

Yet another aspect of the invention is a method for making a transgenic fish in which a retroviral vector is introduced into a cell in a fish embryo and the fish embryo is permitted to develop into a fish. Transgenic fish having a long terminal repeat (LTR) at each end of the retroviral vector insert is screened for.

In addition, transgenic fish having in the genome of at least one cell a retroviral vector insert, the insert having a long terminal repeat (LTR) at each end of the insert, are provided. In preferred embodiments, the retroviral vector insert is a stable, single copy, non-rearranged retroviral vector insert. In certain embodiments, the retroviral vector carries an exogenous DNA sequence to impart a desired characteristic in the transgenic fish. Cells and cell lines derived from such transgenic organisms, are also provided.

The above and other objects, features and advantages of the present invention will be better understood from the following specification when read in conjunction with the accompanying drawings.

Brief Description of the Drawing

FIG. 1 depicts a map of pseudotyped retroviral vector LZRNL.

Detailed Description

This invention provides a method for stably introducing exogenous DNA into the germ line of a fish. A fish embryo having cells is provided. A retroviral vector is introduced into a cell of the fish embryo. The fish embryo is permitted to develop into a fish. A stable chromosomal insertion of the retroviral vector is screened for.

Fish is meant to include, e.g., fresh water fish and salt water fish. Both commercial and non-commercial species of fish are included. Preferably, the fish used has an easily accessible embryo that can be infected with a virus. Preferred fish are, e.g., zebrafish, medaka, chum salmon and rainbow trout. The most preferred fish is zebrafish. The advantage of using zebrafish is that it is a vertebrate animal in which large scale mutagenesis can be performed, and which also has numerous, readily accessible, transparent embryos which are ideal for embryological analysis.

By exogenous DNA is meant DNA that is not normally found in the genome of the fish. A retroviral vector is a vector having at least some retrovirus nucleic acid sequences. A retrovirus is a virus having a genome consisting of two single-stranded RNA molecules. During the infective cycle, the RNA is converted by reverse transcription into single-stranded DNA, which in turn is converted into double-stranded DNA. This double-stranded DNA can become part of the genome of the cell. Any retroviral vector which has a host range that is able to enter the fish cell and integrate into the genome of the fish cell can be used in this invention. Preferably, the retroviral vector is a pseudotyped virus in which the genome, or part of the genome, of one virus is encapsidated by the envelope protein of a different virus. The host range of the pseudotyped virus is often determined by the virus contributing the envelope protein. The virus contributing the envelope protein can be, e.g., from vesicular stomatitis virus (Zavada, J., J. Gen. Virol. 125:183-191 (1972); Zavada, J., Nature New Biology 240:122-124 (1972); Weiss et al., Cold Spring Harbor Symp. Quant. Biol. 39:913-918 (1974); Emi et al., J. Virol. 65:1202-1207 (1991); Burns et al., Proc. Natl. Acad. Sci., USA 90:8033-8037 (1993)), a fish retrovirus from wall eye (Martineau et al., J. Virology 66:596-599 (1992)), or any other virus which has a host range for the particular fish being used. The virus contributing at least one viral gene is preferably a retrovirus. Retroviruses can be, e.g., from mouse, e.g., Moloney virus or other mouse retroviruses, (Mann et al., Cell 33: 153-159 (1983)), chicken, e.g., Rous sarcoma virus or other chicken retroviruses (Petropoulos et al., J. Virol. 65:3728-3737 (1991)), or any other retrovirus whose genome can be incorporated into particles with an envelope that allows them to infect fish cells, and that can integrate their genomes into the fish genome. It is preferable to use a retrovirus which is able to yield high titers. In one preferred embodiment, the pseudotyped retroviral vector particles contain the G protein from vesicular stomatitis virus, and other viral genes from the Moloney murine leukemia virus. This retroviral vector is described in Burns et al., Proc. Nat'l Acad. Sci., USA, 90: 8033-8037 (1993).

In certain embodiments, cell lines which constantly produce retrovirus pseudotype with a particular envelope, e.g., the vesicular stomatitis virus G envelope, can be used as an easy source of the virus.

In certain embodiments, the retroviral vector also contains a reporter gene. By reporter gene is meant a nucleic acid coding sequence whose product is easily assayed. The reporter gene of this invention can be expressed, e.g., in a fish embryo and/or in a transgenic fish. Examples of reporter genes include Escherichia coli lacZ, mouse tyrosinase and jelly fish green fluorescent protein. LacZ can be detected in fixed fish embryos with, e.g., Xgal (obtained from Boehringer-Mannheim,

Indianapolis, IN), resulting in a blue color (Davies et al., J. Mol. Biol. 36:413 (1968); Bayer et al, Development 115:421-426 (1992)), and in live fish embryos and fish with FDG, fluorscein di-ß-D-galactopyranoside (obtained from Molecular Probes, Eugene, Oregon), resulting in a green color. (Nolan et al., Proc. Natl. Acad. Sci., USA, 85:2603-2607 (1988); Lin et al., Developmental Biology 161:77-83 (1994)). Introduction of the tyrosinase gene into an albino fish embryo which lacks tyrosinase, causes the fish to acquire pigmentation. (Matsumoto et al., Pigment Cell Research 5:322-327 (1992)); Beermann et al., EMBO J. 9:2819-2826 (1992)). Green fluorescent protein can be detected in fish embryos and transgenic fish by viewing under a fluorescence microscope with an appropriate filter. (Chalfie et al., Science 263:802-805 (1994)). Preferably, the reporter gene is under a strong promoter or a tissue-specific promoter.

In certain other embodiments, the reporter gene is used in a gene trap retrovirus construct. By gene trap is meant a reporter gene on a piece of DNA or in a viral genome that can only be expressed after the DNA or viral genome integrates into an active gene in the host cell, in this case a fish cell. (O'Kane et al., Proc. Natl. Acad. Sci. USA, 84:9123-9127 (1987); Chang et al., Virology 193:737-747 (1993)). Gene trap vectors are particularly useful for identifying insertions of the retroviral vector into active genes.

In other embodiments, the retroviral vector carries an exogenous DNA sequence, e.g., an exogenous gene, part of an exogenous gene, or any other DNA sequence, which results in a desired characteristic being bestowed upon the fish.

The retroviral vector is introduced into a fish embryo. Fish embryos are meant to include zygotes, and early, mid and late stage embryos. For example, introduction can be into an embryo with about 1 to about 1000 cells, about 1000 to about 2000 cells, about 2000 to about 4000 cells, about 4000 to about 8000 cells, or greater than about 8000 cells. Preferably, the retroviral vector is introduced into the blastoderm, in a cell from a blastula stage embryo. Introduction into a cell that is destined to become the germ line is preferred. The fish embryo is then permitted to develop into a fish. Introduction of the retroviral vector can also be into a fish, preferably a very young fish.

The retroviral vector can be introduced into a cell by any process which results in nucleic acid uptake, e.g., injection, electroporation or retroviral infection. Preferably, introduction is by injection. Most preferably, introduction is by injection of virus among the fish cells of an embryo, resulting in infection of the cells.

In certain embodiments, the chimera method of introducing retroviral sequences, described in Lin et al., Proc. Natl. Acad. Sci. USA, 89:4519-4523 (1993), is used. Cells from about the 1000-2000 cell stage from wild type embryos are infected with a retrovirus in vitro. These infected

cells are introduced, e.g., transplanted, into albino, i.e., pigmentless, fish embryos at about the 1000 to about the 4000 cell stage, and permitted to develop into fish. The fish are mated. If infected donor cells from wild type pigmented embryos formed part of the germ line in the recipient albino embryos, the fish are germ line chimeras. When mated to albinos they will produce pigmented offspring infected with virus.

Another method of retroviral vector introduction is to inject cells that are chronically producing virus into, e.g., the blastoderm of developing fish embryos so that these virus-producing cells infect the germ line of the fish.

Other methods include, e.g., direct injection of the virus into the gonad of the fish, preferably a very young fish; introduction preferentially into primordial germ cells of the fish; or introduction into embryonic stem cell equivalents of fish.

The physical form of the nucleic acid that is introduced into the cell includes, e.g., viral RNA or proviral DNA in various states of completion of synthesis. The nucleic acid molecules can be intact or can contain one or more nicks.

Preferably, the retroviral vector is introduced into the cell in the presence of polybrene. The concentration of polybrene preferably is about 2 ?m?g/ml to about 20 ?m?g/ml, and most preferably is about 8 ?m?g/ml.

Examples 1-6 illustrate that this invention results in high-frequency infection of a fish germ line with a retroviral vector. That infection of a fish embryo with a retroviral vector would be able to result in germ line transmission of integrated proviral DNA was a very unexpected result. Since fish embryos, e.g., zebrafish, develop very rapidly at 28°C, while murine retroviruses generally require over 6 hours at 37°C to synthesize and integrate proviral DNA (Roe et al., EMBO J., 12: 2099 (1993)), it was not expected that germ line transmission could be obtained efficiently or at all.

In certain embodiments, introduction of the retroviral vector can provide the fish with a desired characteristic such that the fish, or a descendent of the fish, has that desired characteristic. Examples of a desired characteristic include enhanced and/or novel nutritional value, disease resistance, growth enhancement, e.g., faster growth, increased body size or increased litter size, or production of a desired protein. By desired protein is meant a protein that bestows a desired trait on the fish in which it is produced or a protein which when isolated from the fish is desirable for uses outside of the fish. The desired protein may be produced in a specific tissue, a subset of tissues or in a wide range of tissues. Examples of desired proteins include proteins which correct an abnormal condition in the fish or therapeutic proteins for the fish or some other animal.

In other embodiments, introduction of the retroviral vector can create an insertional mutation

in the fish such that the fish, or a descendent of the fish, has that mutation. Such mutated genes are readily cloned because the inserted DNA serves as a tag, i.e., a probe, for cloning them. (Soriano et al., Genes and Dev. 1:366-375 (1987)). Retroviruses are ideal insertional mutagens because they insert their genes cleanly into DNA with a predicted proviral structure. (Varmus et al., pp 369-512, In R. Weiss et al., RNA Tumor Viruses, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1982)). Genes affecting any process of interest that can be detected can be identified by such insertional mutagenesis. This includes, e.g., genes for the development of structures and genes for function. For example, mutants can be found in the general body plan of the fish, in the brain and spinal cord, in the gut, thymus, pancreas, kidney, in the heart and in heart function and blood formation, in nerves, muscles and nerve-muscle transmission, in the ears and eyes, e.g., in the ability to see, in the projection of retinal axons to the tectum of the brain, in motility, e.g., in the ability to swim properly, in circadium rhythm and cell death. Mutant fish with tumorous growths and mutant fish whose cells grow better or worse in culture can also be obtained.

Screening for stable chromosomal insertion of the retroviral vector includes, e.g., visual observation, chemical analysis or biological analysis. Screening can be done in the fish embryo, in the fish, or in the F1 progeny of the fish. Preferably, screening for a stable chromosomal insertion of the retroviral vector is followed by screening for the mutation.

In general, it can be determined if an insertion event has occurred by restriction endonuclease digestion and Southern blot hybridization to detect predicted fragments, those internal to the viral genome and also junction fragments between the provirus and host DNA. PCR analysis can also be used to determine whether integration has taken place. For example, Mendelian inheritance of viral nucleic acid sequences, detected by PCR analysis of the offspring of a transgenic fish, provides strong evidence for integration. Methods for carrying out these techniques are well-known to those skilled in the art and can also be found in Varmus et al., pp. 369-512, In R. Weiss et al., RNA Tumor Viruses, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1982); Sambrook, Fritsch & Maniatis, Molecular Cloning, A Laboratory Manual, 2nd ed., pp 14.1-14.35, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

Certain other screens can be done by visual observation in a dissecting microscope. (Mullins et al., Current Biol. 4:189-202 (1994)). Many organ rudiments can be seen in this way within 24 hours after eggs are fertilized. Other screens include, e.g., in situ hybridization, e.g., using gene pr bes for genes expressed specifically in particular organs. (Schulte-Merker et al, Development 116:1021-1032 (1992)). Screens can also be done using stains or antibodies, e.g., to specific cell types (Schulte-Merker et al., Development 116:1021-1032 (1992)), or by injecting dyes, e.g., that

track axons or neurons. (Simon et al., Developmental Biol. 162:384 (1994)). Functional screens include, e.g., those for motility, sight, sound, smell or circadium rhythm. (Mullins et al., Current Biol. 4:189-202 (1994)).

In embodiments in which a reporter gene is present on the retroviral vector, assays can be performed for the presence of the reporter gene product. Examples of reporter genes and possible assays for them are described above.

Other types of screens include, e.g., histochemical assays, immunohistochemical assays, enzymatic assays, protein purification, in situ hybridization methods in whole fish, tissue sections, cell homogenates or single cells, RNA hybridization or RNAse protection assays. For the transgenic fish of this invention, assays can be performed using samples from different tissues.

This invention also includes a method for making a transgenic fish in which a retroviral vector is introduced into a cell in a fish embryo and the fish embryo is permitted to develop into a fish. In certain embodiments, the retroviral vector is introduced into a fish, preferably a very young fish. Transgenic fish having a retroviral vector insert are screened for, the retroviral vector insert having a long terminal repeat (LTR) (Varmus et al. pp 369-512, In R. Weiss et al., RNA Tumor Viruses, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1982)) at each end of the retroviral vector insert. In certain embodiments, the retroviral vector insert is a stable, single copy, non-rearranged retroviral vector insert. The transgenic fish obtained from this method are also included in this invention. By transgenic fish is meant a fish that gains new genetic information from the introduction of exogenous DNA into its own, or into an ancestor's germ line.

This invention also includes an isolated fish cell from a transgenic fish or fish embryo, or a descendent of the fish cell, which has a retroviral vector insert having a long terminal repeat (LTR) at each end of the retroviral vector insert. In preferred embodiments the genome has a stable, single-copy, non-rearranged retroviral vector insert in its genome.

This invention further includes a transgenic fish having in the genome of at least one cell, a retroviral vector insert, the insert having a long terminal repeat (LTR) at each end of the retroviral vector insert. Preferably, the retroviral vector insert is a stable, single-copy, non-rearranged retroviral vector insert. In certain embodiments, the retroviral vector carries an exogenous DNA sequence to impart a desired characteristic in the transgenic fish. In other embodiments the retroviral vector insert is in a gene of the fish so as to result in a mutation in the gene. Variations include a transgenic fish in which all f the cells of the fish have the insert, and transgenic fish which are chimeric fish. By chimeric fish is meant a fish in which some of its cells have gained new genetic information from the introduction f exogenous DNA into the fish, or an ancestor of the

fish, preferably at an embryonic stage. A cell, or a descendent of this cell, derived from a somatic cell from such a transgenic fish, and a cell line derived from such a somatic cell are also included in this invention.

This invention has many uses. Retroviral vector integration is a powerful method for making useful transgenic fish both for basic research and for commercial use. Many fish are commercially important. The ability to make transgenic fish by introducing genes by retroviral vector infection into fish embryos is very useful to the fishing industry. For example, introducing a growth hormone gene and/or a disease resistance gene, will lead to faster growing, healthier fish for fish farming.

In addition, given that fish is an ideal vertebrate animal in which to study development, one can use the retroviral vector insertion methods of this invention to genetically define all genes which, when mutated one at a time by retroviral vector insertion, cause a defect in any process for which one has an assay. A commercial potential for this invention thus is as a gene-finding device. Key genes in processes such as diabetes, heart disease, cancer, growth, and cell death are of particular utility. Often genes that are identified in developmental processes are the same as the genes for these diseased states. Proteins coded by such key genes are important targets for drug design, or can be useful therapeutic reagents themselves.

EXAMPLES

Example 1: Generation of Transgenic Zebrafish By Retroviral Vector Infection of Embryos

This example illustrates the generation of transgenic zebrafish resulting from injection of a pseudotyped retroviral vector, LZRNL(G) virus, into blastula stage embryos.

LZRNL(G) contains the G protein from the vesicular stomatitis virus (VSV) and other viral genes from the Moloney murine leukemia virus (MoMLV). (See FIG. 1). Burns et al., Proc. Nat'l Acad. Sci., USA, 90: 8033-8037 (1993). LZRNL(G) differs from LSRNL(G) described in the Burns et al. paper in that LZRNL(G) carries the lacZ gene, whereas LSRNL(G) carries the hepatitis B virus surface antigen. A concentrated stock of the pseudotyped virus LZRNL(G), was generated. A 293-derived cell line stably expressing the MoMLV gag and pol genes as well as the LZRNL genome was transiently transfected with a plasmid encoding the VSV-G gene driven by the human cytomegalovirus promoter. Virus containing supernatant was collected 48-72 hours later and concentrated as described in Burns et al., Proc. Nat'l Acad. Sci., USA, 90: 8033-8037 (1993). This virus was titered on cultured zebrafish cells by infecting an established zebrafish cell line, PAC2. The PAC2 cell line was derived from 24 hour old embryos and was maintained in Lebowitz-15 media supplemented with 15% FBS and 5% zebrafish embryo extract. Clones were

selected in media containing G418. Concentrated stocks of LZRNL(G) were diluted and used to infect NIH 3T3's, and PAC2's for three hours in the presence of 8?m?g/ml of polybrene (obtained from Sigma, St. Louis, MO). Approximately 4 hours after the completion of the infection, the infected cells were trypsinized, serially diluted into both selective media and nonselective media, and plated. The plates were stained with crystal violet 10 days later and the number of colonies were counted. LZRNL contains the neomycin phosphotransferase gene (neo) and thus can confer G418 resistance to infected cells. Control mouse 3T3 cells were infected under the same conditions. The titer of the virus was $6.7x10^6$ cfu/ml on zebrafish PAC2 cells, and $2.5x10^7$ cfu/ml on mouse 3T3 cells.

To generate transgenic zebrafish, LZRNL(G) virus was injected into the blastoderm, among the cells of blastula stage zebrafish embryos, at approximately the 2000-4000 cell stage. Concentrated pseudotyped virus derived from the vector pLZRNL was resuspended in TNE (50 mM Tris-HCl, pH 7.8/130 mM NaCl/1 mM EDTA) containing 8?m?g/ml polybrene. For injection, dechorionated eggs were incubated in Holtfreter's solution at 25-28°C for about 4-5 hours and the blastula-stage embryos were injected with a total of 10-20 nl of virus into multiple locations in each embryo using a glass needle and a dissecting microscope. On the basis of the virus titer on PAC2 cells and the volume injected, it was estimated that at least about 50-100 infectious units were injected into each embryo. The injected embryos were permitted to develop into fish.

Example 2: Germ Line Transmission of Proviral DNA from Retroviral Infection of Zebrafish Embryos

This example illustrates germ line transmission of proviral DNA from retroviral infection of zebrafish embryos.

Injected embryos from Example 1 were raised to sexual maturity, mated, and DNA from 24-hour-old pools of their F1 progeny were tested for the presence of LZRNL sequences by PCR. DNA was extracted from pools of 50-100 F1 embryos at 24 hours of development, or from individual fish by incubation for 4-12 hours at 55°C in a lysis buffer (10 mM Tris-HCl pH 8.0/10mM EDTA/100 mM NaCl/.4% SDS/.200 ?m?g/ml proteinase K). DNA was precipitated by ethanol and dissolved in TE (pH 8.0). Appr ximately 10 ng of DNA was used for PCR using AmpliTaq Polymerase (obtained from Perkin Elmer, Cetus, Branchburg, N.J.). The reaction was carried out at 94 C/30", 60°C/45", 72°C/60" for 32 cycles with an initial 2 minute denaturation step at 94°C. The two primers used to detect the presence of LZRNL DNA sequence yielded a 300 bp PCR product. The 5' primer (P1)

was 5'GGGAATGTAGTCTTATGCAATAC3'. (Seq. ID No. 1). The 3' primer (P2) was 5'GCACACCAATGTGGTGAATGGTC3'. (Seq. ID No. 2). See FIG. 1 in which the PCR primers used to identify the transgenic fish are indicated with arrows. A pair of internal control primers homologous to the zebrafish Wnt5A gene, 5'CAGTTCTCACGTCTGCTACTTGCA3' (Seq. ID No. 3) and 5'ACTTCCGGCGTGTTGGAGAATTC3' (Seq. ID No. 4) or to the ZF21 gene, 5'GAACTAGCAGCAGCGCTATGAAC3' (Seq. ID No. 5) and 5'ATGTAGTTTCCTCATCCAAGGG3' (Seq. ID No. 6), was included in each reaction. The products of the PCR reaction were run on a 2% agarose gel. A control band, indicating that the PCR reactions took place properly, was seen in each sample lane. A band of a different size, indicating the presence of the transgene, namely the viral sequences, was present only in the DNA from offspring of transgenic fish.

In total, 8 out of 51 fish examined showed germ line transmission of the retroviral sequences. Because only 50-100 F1 embryos were collected to test for germ line transmission, founders which transmitted proviral sequences to less than 1% of their offspring may have been overlooked.

Example 3: Founder Zebrafish From Retroviral Vector Infection of Embryos Have Mosaic Germ Lines

This Example illustrates that the founder zebrafish generated from retroviral vector infection of embryos from Example 1, have mosaic germ lines.

Because the virus was injected into blastula-stage embryos containing a large number of potential target cells, the embryos were very likely to be mosaic for the presence of integrated viral sequences. To determine if the founder fish had mosaic germ lines, individual F1 progeny from each founder were analyzed by PCR for the presence of proviral DNA. As shown in Table 1, all eight founders did indeed have mosaic germ lines and transmitted proviral DNA to less than 5% of their F1 progeny.

TABLE 1

Mosaicism of Germ Line Transmission of Proviral DNA from Founders to the F1 Generation

Founder	Transgenic F1's	Mosaicism
m4	6/306	2.0%
f6	3/128	2.3%
f12	2/86	2.3%
f13	1/20	5.0%

f36	2/110	1.8%
f44	1/60	1.7%
f45	2/50	4.0%
m49	1/115	0.9%

Example 4: Mendelian Germ Line Transmission of Proviral DNA

This example illustrates that the proviral DNA integrated into the zebrafish genome resulting from retroviral vector infection of embryos, is transmitted in a Mendelian fashion.

Live transgenic F1 fish generated from the Examples above, were identified by isolating genomic DNA from caudal fin clips and using PCR to test for the presence of the viral transgene. Two transgenic F1's identified in this way were then mated to non-transgenic fish and individual F2 embryos were screened by PCR. If a transgenic F1 fish contained an integrated provirus, that provirus should have been transmitted to 50% of the F2 progeny. Two F1 fish were thus tested. The first F1 tested transmitted the transgene to 11 out of 25 of its F2 progeny (44%) and the second F1 transmitted the transgene to 8 out of 17 of its F2 progeny (47%). These frequencies are consistent with Mendelian transmission and support the conclusion that the proviral DNA was integrated into the zebrafish genome.

Example 5: Retroviral DNA Integration into a Transgenic Zebrafish Chromosome Retains the Linear Organization of the Retroviral Genome

This examples illustrates that the retroviral vector sequences integrated as single-copy, non-rearranged inserts, in the expected linear arrangement in the infected zebrafish cells and transgenic zebrafish.

Typically, retroviral DNA integrates into a host chromosome as a single copy in a manner that maintains the linear organization of its viral genome. (Varmus, H.E., Science, 216: 812 (1982)). To confirm that the proviral sequences from Example 1 were integrated in the expected arrangement in the infected fish cells and transgenic animals, Southern blot analysis was performed. Genomic DNA from PAC2 clones or individual fish was digested with the appropriate restriction enzymes, was run through a 0.8% agarose gel using electrophoresis, and was then transferred to Hybond N$^+$ membrane (obtained from Amersham, Arlington Heights, IL). Hybridizations were carried out as suggested by the vendor in the presence of a probe labeled with ^{32}P-?a?-ATP using a random primed labeling kit (obtained from Boehringer Mannheim, Indianapolis, IN). The probe was derived from pLZRNL by a single Cla I digestion (FIG. 1).

Genomic DNA from two clones of cultured PAC2 cells infected with LZRNL(G), as well as from the transgenic F1 progeny of two different founders (m4 and f13), was digested with the restriction enzyme Dra I. In addition, genomic DNA from the two infected PAC2 clones and from

the transgenic F1 progeny of all eight founders was digested with Hind III. Dra I does not cleave within the LZRNL sequence and should yield one or more fragments, depending upon the number of integrations, with sizes larger than the proviral genome (6.6-Kb). Hind III cleaves twice within the LZRNL sequence (see FIG. 1) and is expected to yield a 3.7-Kb internal fragment for all insertions, and two junction fragments with sizes dependent upon the presence of Hind III sites in the surrounding genomic sequences. The Cla I fragment of pLZRNL containing lacZ, RSV, and neo sequences, was used as the hybridization probe (FIG. 1), and was expected to hybridize to both the internal 3.7 Kb Hind III fragment and to the 3' junction fragment.

Samples digested with Dra I revealed only single bands of variable size, indicating the presence of single copies of integrated provirus. In the case of Hind III digests, a 3.7-Kb fragment of LZRNL was detected in the genomic DNA of the transgenic fish and the PAC2 clones. In addition, each lane had a second band of variable size, presumably representing the 3' junction fragment. The variation in size between these junction fragments indicates that the sites of proviral integration were distinct in each case.

Example 6: Multiple Retroviral Vector Integration Events Can Occur in Each Infected Zebrafish Embryo

This example illustrates that multiple retroviral integration events can occur in each infected zebrafish embryo.

The fact that approximately 50-100 infectious units were injected into each embryo in Example 1 suggested that multiple integration events could readily have occurred in each embryo. To determine whether or not a founder fish transmitted more than one proviral integration through its germ line, the insertions of three different transgenic F1's from a single founder (m4) were compared by means of Southern blot analysis. Genomic DNA was digested with Hind III and probed with the Cla 1 fragment of pLZRNL. As expected, all three F1's had the internal 3.7 Kb band as well as a 3' junction fragment. A comparison of the junction fragments, however, indicated that two of the fish had the same insertion, but that the third fish had an insertion different from that of the other two. This result demonstrates that for this founder, at least two insertions had been transmitted independently through the germ line.

Those skilled in the art will be able to ascertain, using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: METHOD FOR RETROVIRAL VECTOR INSERTION IN FISH

(iii) NUMBER OF SEQUENCES: 6

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: NOT AVAILABLE
(B) FILING DATE: FILED HEREWITH

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/281,706
(B) FILING DATE: 27-JULY-1994

(viii) ATTORNEY/AGENT INFORMATION:
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(C) REFERENCE/DOCKET NUMBER: M0828/7007

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGAATGTAG TCTTATGCAA TAC 23

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCACACCAAT GTGGTGAATG GTC 23

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CAGTTCTCAC GTCTGCTACT TGCA 24

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACTTCCGGCG TGTTGGAGAA TTC 23

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAACTAGCAG CAGCGCTATG AAC 23

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATGTAGTTTC CTCATCCAAG GG 22

CLAIMS

1. A method for stably introducing exogenous DNA into the germ line of a fish, comprising:

providing a fish embryo having cells;

introducing a retroviral vector into a cell of said fish embryo;

permitting said fish embryo to develop into a fish; and

screening for stable chromosomal insertion of said retroviral vector.

2. The method of claim 1 wherein said fish is a zebrafish.

3. The method of claim 1 wherein said retroviral vector is a pseudotyped virus having a host range so as to be able to enter said cell of said fish embryo and integrate into the genome of said cell.

4. The method of claim 1 wherein said retroviral vector is packaged in particles having an envelope from a first virus.

5. The method of claim 4 wherein said first virus is vesicular stomatitis virus.

6. The method of claim 1 wherein said retroviral vector comprises a viral gene derived from a second virus, said second virus being a retrovirus.

7. The method of claim 6 wherein said retrovirus is Moloney murine leukemia virus.

8. The method of claim 1 wherein said retroviral vector carries an exogenous DNA sequence to impart a desired characteristic.

9. The method of claim 1 wherein said retroviral vector comprises a reporter gene.

10. The method of claim 9 wherein said reporter gene is selected from the group consisting of lacZ, tyrosinase and green fluorescent protein.

11. The method of claim 1 wherein said fish embryo has a blastoderm and said introducing of said retroviral vector is into said blastoderm.

12. The method of claim 1 wherein said fish embryo is a blastula stage embryo, and said introducing of said retroviral vector is into a cell from said blastula stage embryo.

13. The method of claim 1 wherein said fish embryo comprises about 1 to about 1000 cells.

14. The method of claim 1 wherein said fish embryo comprises about 1000 to about 2000 cells.

15. The method of claim 1 wherein said fish embryo comprises about 2000 to about 4000 cells.

16. The method of claim 1 wherein said fish embryo comprises about 4000 to about 8000 cells.

17. The method of claim 1 wherein said fish embryo comprises greater than about 8000 cells.

18. The method of claim 1 wherein said introducing of said retroviral vector is by injection into said fish embryo.

19. The method of claim 1 wherein said introducing of said retroviral vector is done in the presence of polybrene.

20. The method of claim 19 wherein said polybrene is at a concentration of about 2 ?m?g/ml to about 20 ?m?g/ml.

21. The method of claim 19 wherein said p lybrene is at a concentration of about 8 ug/ml.

22. The method of claim 1 wherein said introducing of said retroviral vector comprises introducing said retroviral vector in vitro into a cell from a first fish embryo and then introducing said cell with said retroviral vector into a second fish embryo.

23. The method of claim 19 wherein said first embryo is wild type and said second fish embryo is albino.

24. The method of claim 1 wherein said retroviral vector is introduced so as to create a mutation in said fish, and wherein said fish, or a descendent of said fish, has said mutation.

25. The method of claim 1 wherein said insertion of said retroviral vector provides said fish with a desired characteristic and wherein said fish, or a descendent of said fish, has said desired characteristic.

26. The method of claim 1 wherein said screening is done in said fish embryo.

27. The method of claim 1 wherein said screening is done in said fish.

28. The method of claim 1 wherein said screening is done in F1 progeny of said fish.

29. A method for stably introducing exogenous DNA into the germ line of a fish, comprising:

providing a fish having cells;

introducing a retroviral vector into a cell of said fish;

screening for stable chromosomal insertion of said retroviral vector.

30. A method for making a stable insertional mutation in a fish, comprising:

introducing a retroviral vector into a cell in a fish embryo;

permitting said fish embryo to develop into a fish;

screening for stable chromosomal insertion of said retroviral vector; and

screening for a mutation.

31. A method for treating a fish cell in an embryo so as to cause an insertion of a retroviral vector in a chromosome of said fish cell, comprising:

introducing into said fish cell in an embryo a retroviral vector; and

screening said fish cell, or a descendent of said fish cell, for insertion of said retroviral vector into a chromosome of said fish cell.

32. A method for making a transgenic fish, comprising:

introducing a retroviral vector into a cell in a fish embryo;

permitting said fish embryo to develop into a fish; and

screening for a transgenic fish having a retroviral vector insert, said retroviral vector insert having a long terminal repeat (LTR) at each end of said retroviral vector insert.

33. The method of claim 32, wherein said retroviral vector insert is a stable, single copy, non-rearranged retroviral vector insert.

34. The transgenic fish obtained from claim 32.

35. An isolated fish cell from a transgenic fish or fish embryo, or a descendent of said fish cell, having a genome, said genome having a stable, single-copy, non-rearranged retroviral vector insert.

36. A transgenic fish having in the genome of at least one cell a retroviral vector insert, said retroviral vector insert having a long terminal repeat (LTR) at each end of said retroviral vector insert.

37. The transgenic fish of claim 36, wherein said retroviral vector insert is a stable, single copy, non-rearranged retroviral vector insert.

38. The transgenic fish of claim 36 wherein the retroviral vector carries an exogenous DNA sequence to impart a desired characteristic.

39. The transgenic fish of claim 36 wherein said retroviral vector insert is in a gene of said fish so as t result in a mutation in said gene.

40. The transgenic fish of claim 36 wherein said fish is a chimeric fish.

41. The transgenic fish of claim 36 wherein all of the cells of said fish have said insert.

42. A cell, or a descendent of said cell, derived from a somatic cell obtained from said transgenic fish as claimed in claim 36.

43. A cell line derived from a somatic cell obtained from said transgenic fish as claimed in claim 36.

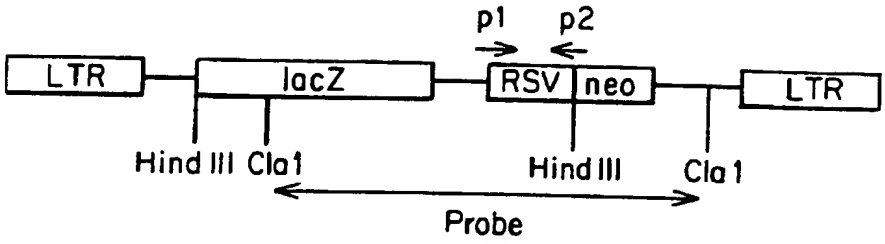

Fig. 1

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US95[illegible]88

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01K 61/00; C12N 5/00, 15/00
US CL :119/215, 217; 435/172.1, 172.3, 240.2, 320.1; 800/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 119/215, 217; 435/172.1, 172.3, 240.2, 320.1; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, ASFA, EMBASE, MEDLINE, DERWENT, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 263, issued 11 February 1994, Chalfie et al, "Green fluorescent protein as a marker for gene expression", pages 802-805, see the entire document.	9, 10
X --- Y	Molecular Marine Biology and Biotechnology, Volume 2, Number 3, issued 1993, Ivics et al, "Enhanced incorporation of transgenic DNA into zebrafish chromosomes by a retroviral integration protein", pages 162-173, see the entire document.	1,2,8,9,13,18, 24-27,30,31 ---------- 1 4 17,28,29,32-43

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 OCTOBER 1995	03 NOV 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer BRUCE CAMPELL
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/09588

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Proceedings of the National Academy of Science, USA, Volume 90, issued September 1993, Burns et al, "Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: Concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells", pages 8033-8037, see the entire document.	35 --- 1-34,36-43
Y	Proceedings of the National Academy of Science, USA, Volume 89, issued May 1992, Lin et al, "Production of germ-line chimeras in zebrafish by cell transplants from genetically pigmented to albino embryos", pages 4519-4523, see the entire document.	22,23,34,36-41
X, P	Science, Volume 265, issued 29 July 1994, Lin et al, "Integration and germ-line transmission of a peudotyped retroviral vector in zebrafish", pages 666-669, see the entire document.	1-43